Research Article

Hesperidin methyl chalcone alleviates spinal tuberculosis in New Zealand white rabbits by suppressing immune responses

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Objective: Spinal tuberculosis (ST) refers to tuberculosis resulted from infections of *Mycobacterium tuberculosis* (*Mtb*) in the spinal cord. Hesperidin methyl chalcone (HMC) is a flavonoid derivative from citrus fruits with anti-inflammatory properties. We aimed to investigate the efficacy of HMC in treating ST in New Zealand white rabbit model.

Design and Setting: Rabbits were infected in the sixth lumbar vertebral bodies with or without *Mtb* strain H37Rv followed by treatments with HMC.

Outcome Measures: 10 weeks post treatments, the adjacent vertebral tissues were examined by hematoxylin–eosin staining. The expression levels of transcription factor κB (NF-κB) p65 and monocyte chemoattractant protein-1 (MCP-1/CCL2) in lymphocytes were determined using reverse transcription quantitative real-time PCR (RT-qPCR), Western blot and enzyme-linked immunosorbent assays (ELISA). The serum levels of interleukin (IL)-2, IL-4, IL-10 as well as interferon (IFN)-γ were also assessed using ELISA. Western blot was used to determine the effects of HMC on the phosphorylation of IKKα/β, p65, and IκBα in the signal transduction of NF-κB pathways.

Results: HMC significantly attenuated the granulation in adjacent vertebral bone tissues. The expression of p65, IL-4, IL-10, and MCP-1 was reduced. The NF-κB pathway was suppressed, in which the phosphorylation of IκBα, IKKα/β, and p65 was inhibited whereas the relative level of IκBα was increased.

Conclusion: HMC could serve as a therapeutic option to effectively inhibit granulomas formation through downregulation of MCP-1, IL-4, IL-10, and NF-κB in the treatment of ST.

Keywords: Spinal tuberculosis, Hesperidin methyl chalcone, Granuloma, Inflammatory cytokines, Immune response

Introduction

Spinal tuberculosis (ST), also known as the Pott's disease, results from *Mycobacterium tuberculosis* (*Mtb*) infections inside spinal cavities.[1] ST often starts with back pain, leading to the spread of abscess into spinal cords, which eventually causes spinal collapse, neurologic defects, and even paralysis.[1–3] As one of the oldest illnesses, the earliest record of ST was from 3400 BC Egypt.[4] In the mid-20th century, due to the developments of anti-tuberculosis drugs including rifampicin, streptomycin, and isoniazid, ST was nearly eliminated at the time.[5] However, the emergence of multidrug resistant strains in *Mtb* and the wide-spread of human acquired immune deficiency syndrome have given rise to the recurrence of tuberculosis, particularly in developing countries.[1,5] The main problems now are focused upon the irreversible destruction of bone tissues and neurological defects resulted from chronic inflammation.[6,7] Based on WHO recommendations, various combinations of anti-tuberculosis drugs are employed to treat different subtypes or stages of ST.[8] Anti-inflammatory therapies, however, were not included. Thus, it is of great importance to develop new drugs to alleviate the chronic inflammation in patients with ST.

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The formation of granulomas, which are aggregates of well-organized immune cells (mostly lymphocytes and macrophages) enclosing the non-absorbable stimulus, is a hallmark of tuberculosis.[9] Granulomas was once regarded as a protective structure in the eradication of *Mtb*; however, recent progress has shown that granulomas play important roles in the dissemination of pathogens.[9–11] *Mtb* can survive for years within granulomas, which is a major cause of clinical latency and the recurrence of the disease.[12]

The granulomas formation starts with the upregulated secretion of proinflammatory cytokines and chemokines.[13] Monocyte chemoattractant protein-1 (MCP-1/CCL2) is a widely studied chemokine from the C-C chemokine family. The production of MCP-1 is suggested in the recruitment as well as activation of T-lymphocytes and monocytes during the granulomas formation.[14] Prior studies have shown that the susceptibility to tuberculosis is determined by the polymorphism of MCP-1.[15] Nuclear factor κB (NF-κB), a multifunctional transcription factor, controls the expression of several proinflammatory cytokines and chemokines. Therefore, it is a crucial pathway in inflammation. Tumor necrosis factor (TNF), the activator of NF-κB, has been demonstrated as an essential factor to restrict the growth of bacteria inside granulomas.[12,16,17] In a previous study, it was shown that the expression of NF-κB and MCP-1 are both elevated in New Zealand rabbits carrying ST,[18] suggesting both proteins can be pivotal targets in attenuating the inflammation in ST.

Hesperidin possesses anti-inflammatory properties, but as many other flavonoids, it is poorly absorbed in the small intestine due to its low water solubility.[19] Hence, improving water solubility by methylation could enhance the bioavailability, tissue distribution, and metabolic stability.[20] The methylation of hesperidin under alkaline conditions generates hesperidin methyl chalcone (HMC) with greater water solubility.[21] Like its parental compound hesperidin, HMC too exhibits potent anti-inflammatory properties in several disease models. For instance, HMC was shown in a mouse pain model to reduce inflammation and inflammatory pain by inhibiting oxidative stress, cytokine production, and NF-κB activity.[22] Similarly, in a mouse model of ultraviolet B irradiation-induced skin damage, HMC could suppress oxidative stress and inflammation[23] by inhibiting cytokine productions of cytokines including TNF-α, IL-1β, IL-6, and IL-10.[24] However, there has been no report on the effect of HMC on the immune responses during ST.

In the present study, New Zealand white rabbits infected with *Mtb* at spinal cord were treated with HMC. The expression levels of NF-κB and MCP-1 as well as the serum levels of inflammatory cytokines were examined. We presented evidence that HMC has the potential to serve as an effective drug in attenuating the chronic inflammation in ST.

Methods

Cell lines and reagents

H37Rv standard strain was cultured on Roche culture medium (Jinan Cell Biology, Shandong, China) for two weeks. Colonies with healthy growth were picked out and dissolved in sterilized standard saline to a final concentration of 1 mg mL^{-1}. HMC (purity 98%) was purchased from Stanford Chemicals (Lake Forest, CA, USA).

New Zealand white rabbit ST model

New Zealand white rabbits (3–4 weeks; 3.25 ± 0.25 kg) were randomly divided into three experimental groups (n = 10 each group): (1) sham group; (2) ST group; (3) ST + HMC group. Treatments to rabbits in each group were as follows: rabbits were anesthetized with 2% barbital sodium iodine (20 mg kg^{-1}; Sigma Aldrich, MO, USA), and holes were drilled on the sixth lumbar vertebras of rabbits. Gelfoam sponges soaked in normal saline (sham group) or 0.1 mL H37Rv suspension (ST model rabbits) were placed into the perforations of these rabbits. After suturing the incisions, the ST rabbits were injected daily through an auricular vein, with normal saline as vehicle (ST group) or 30 mg/kg HMC in normal saline (ST + HMC group), respectively. The treatment continued for 10 weeks. After the final injection, tissues surrounding the 5–7 lumbar vertebral bones were collected for hematoxylin–eosin (H&E) staining and peripheral venous blood were harvested for future analyses. HMC dosage was followed according to the previously established protocol.[25] All experiments were approved by the Ethics Committee of Dongzhimen Hospital Affiliated to Beijing University of Chinese Medicine.

Western blot

Lymphocytes in the blood obtained from experimental animals were isolated through lymphocytes separation medium (Mediatech, Manassas, VA, USA), and subsequently sonicated and centrifuged. Proteins in the supernatants of cell lysates were separated through 12% polyacrylamide gel electrophoresis. After SDS-PAGE, the isolated proteins were transferred onto polyvinylidene fluoride membranes (Sigma Aldrich, MO, USA), which were rinsed twice with cold Tris-buffered saline (TBS) buffer and blocked with 3% BSA, followed by incubation with any of anti-p65, anti-p-p65,

anti-MCP-1, anti-IκBα, anti-p-IκBα, anti-IKKα/β, anti-p-IKKα/β, or anti-β-actin primary antibodies (Abcam, Cambridge, MA, USA) diluted in the ratio of 1:1000 for 2 hours, using β-actin (1:2000) as the internal control. The primary antibodies were then rinsed off with cold PBS buffer and membranes were incubated with HRP-conjugated secondary antibody (Abcam) diluted in the ratio of 1:10 000 for 2 hours, followed by reaction with enhanced chemiluminescence (ECL, Thermo Fisher Scientific, Waltham, MA, USA) substrate for 1 minute at room temperature. The final signals on the membrane were visualized through charge-coupled device (CCD, Thermo Fisher Scientific) digital imaging. All the experiments were performed for three times separately.

Enzyme-linked immunosorbent assay

To determine the serum concentration of proinflammatory cytokines and the expression of multiple proteins inside lymphocytes, the serums or supernatants of lymphocyte lysates were serial diluted and three dilutions were loaded into each well of 96-well plates. Five dilutions of standard samples (50 μL, Shenzhen Jingmei Biological Engineering Co., Ltd., China) of desired proteins were loaded as control. The plates were then rinsed with TBS buffer, dried, and blocked with 3% BSA. Primary antibodies (100 μL), same as those used in Western blotting or antibodies of IL-2, IL-4, IL-10, and IFN-γ (Abcam) were added to the plates. After incubation for 1 h, the plates were again washed and incubated with 100 μL HRP conjugate secondary antibodies, and then rinsed twice with TBS buffer and dried. After drying, chromogenic substrates (100 μL, Thermo Fisher Scientific) were added into each well and allowed to react for 30 minutes in dark at room temperature. Stop solution (100 μL, Thermo Fisher Scientific) was then added to the plate. The absorbance was examined using Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific) at the wavelengths of 450 and 550 nm, and normalized to the standard samples. All of the experiments were repeated three times.

Real-time quantitative reverse transcription polymerase chain reaction

The relative expression levels of p65 and MCP-1 in lymphocytes were determined using RT-qPCR. The mRNAs extracted from cell lysates of lymphocytes were reverse transcribed into first-line cDNA using Quant Reverse Transcriptase (Tiangen Biotech, Beijing, China). Quantitative PCR using FastFire qPCR PreMix (Probe; Tiangen) in ABI7500 Real-time PCR (Thermo Fisher Scientific) system was then conducted. The expression of GAPDH was used as the internal control. The relative expression of each mRNA was normalized to that in the control group. All of the experiments were repeated for at least three times. Primers used were listed as follows: p65 forward 5′-ACA TCC ATG CGG AGA ACG AGG AG-3′, reverse 5′-AGT GCT GCG AGT GAG TCA AGA GG-3′; Mcp-1 forward 5′-AGG TGT AAA GGC AGG TGT GGT-3′, reverse 5′-AGC AGA GTG GGT GGA TTC TTC-3′; GAPDH forward 5′-CGC CTG GAG AAA GCT GCT A-3′, reverse 5′-ACG ACC TGG TCC TCG GTG TA-3′.

Statistical analysis

All data were analyzed with SPSS 22.0 system (IBM, Armonk, NY, USA), and presented as mean ± standard deviation (SD). The differences between two groups were determined using Student's T-tests and single-factor variance analysis (ANOVA). Chi-square test was employed to analyze data shown in percentage. $P < 0.05$ was considered statistical significance.

Results

HMC attenuated the formation of the granulomatous inflammation with Langhans giant cells surrounding the caseous necrosis

To assess the effects of HMC on the inflammatory symptoms in ST, 30 rabbits were assigned to three groups: 20 rabbits were infected with H37Rv cells from *M. tuberculosis* strain, and another 10 rabbits without infection were parallel treated as the sham control group. Starting the day of infection, ST rabbits received a daily dose of 30 mg/kg HMC or vehicle (standard saline) administered through an auricular vein. After 10 weeks of continuous treatments, all rabbits were anesthetized and tissues from adjacent vertebral bone tissues were collected and H&E stained for analysis. Compared with the specimens from the sham controls, tissues from the ST rabbits were darker and contained plenty of granulomatous, in which caseous necrosis was surrounded by Langhans giant cells (Figure 1A and B). In the ST + HMC group, tissues presented a staining pattern similar to that of healthy tissues, indicating that daily treatment with 30 mg/kg HMC effectively decreased granulomatous formation (Figure 1C).

HMC inhibited the expression of MCP-1 and NF-κB

One way to determine the extent of inflammation was to evaluate the expression of biomarkers in inflammatory responses, which were NF-κB and MCP-1 in the

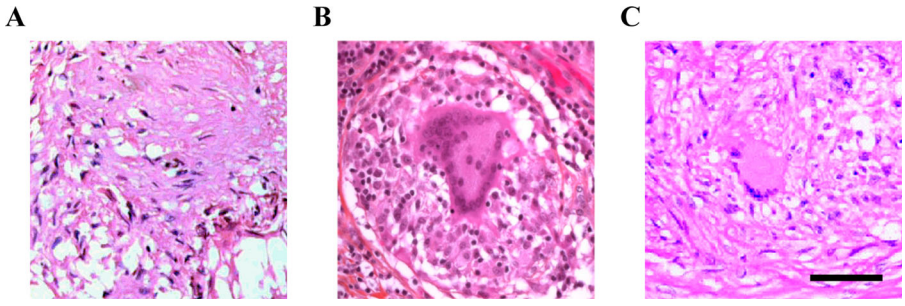

Figure 1 Hematoxylin–eosin staining of adjacent vertebral bone tissues of rabbits. (A) Sham-operated control group. (B) ST experimental group. (C) ST experimental with 30 mg/kg HMC treatment. Scale bar 100 μm.

present study. We first examined their relative messenger levels using RT-qPCR. The relative expressions of both proteins were elevated in ST rabbits, and such increase was prevented by the treatments of HMC (Figure 2A). We then assessed the expression of NF-κB and MCP-1 at the protein level through Western blot and ELISA (Figure 2B and C). The effects of preventing the elevation of the expression of p65 and MCP-1 were significant in ST + HMC group. It was obvious that the expressions of both proteins were relatively low in healthy tissues from sham group, and the infection with *Mtb* upregulated their expression to almost 2 to 3 times of baseline, which was restored to levels similar to the sham group by HMC treatment (Figure 2D).

HMC suppressed the secretion of proinflammatory cytokines

Proinflammatory cytokines are critical proteins in both innate and acquired immune responses. We measured the serum levels of proinflammatory cytokines IFN-γ, IL-2, IL-4, and IL-10 through ELISA. In general, *Mtb* infection stimulated the secretion of all of the above-mentioned cytokines with the exception of IL-2 in experimental groups compared with those of the sham

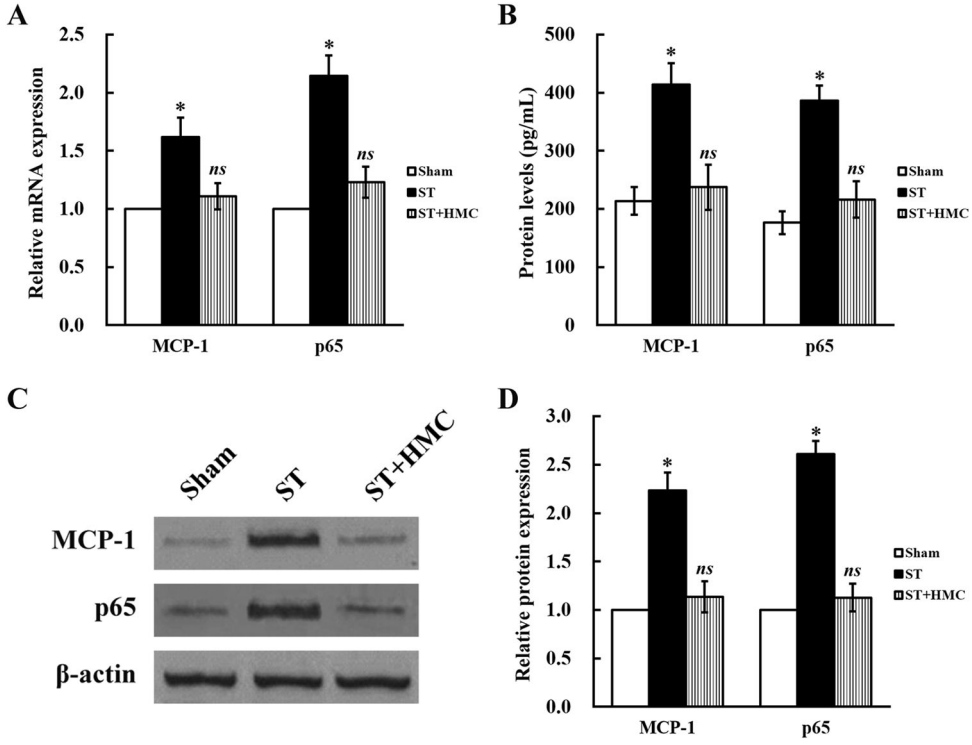

Figure 2 The effects of HMC on the expression of MCP-1 and NF-κB in lymphocytes. (A) Relative mRNA expression of MCP-1 and NF-κB. (B) Protein expressions of MCP-1 and NF-κB detected by ELISA. (C) Protein expressions of MCP-1 and NF-κB were analyzed by Western blot. (D) Protein expressions of MCP-1 and NF-κB by Western blot were quantified. *P<0.05 compared to the sham and ST + HMC groups; ns: P>0.05 compared to sham group (*n* = 10 each group).

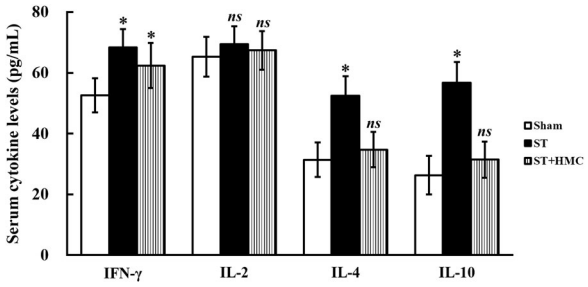

Figure 3 The effects of HMC on serum levels of IFN-γ, IL-2, IL-4 and IL-10 detected by ELISA. *P<0.05; ns: P>0.05, compared to sham group (n = 10 each group).

group (Figure 3). HMC treatment significantly suppressed the serum levels of IL-4 and IL-10 in comparison to the ST rabbits. However, the effects of HMC on IFN-γ and IL-2 were not obvious, since little differences were observed when comparing their expression in ST + HMC group with ST group.

HMC inhibited the phosphorylation and promoted the expression of IκBα

We then investigated the role of NF-κB pathway in the effects of HMC against inflammation in ST. IκBα is an important inhibitor of NF-κB transcription factors. The presence of IκBα and phosphorylated-IκBα (p-IκBα) were examined through Western blot. Compared to the sham group, the protein level of IκBα was markedly reduced in the ST group, whereas p-IκBα level was increased (Figure 4A). Treatment with 30 mg/kg of HMC significantly increased the expression of IκBα and decreased p-IκBα to levels similar to those in the sham group. The expression levels of IκBα and p-IκBα were further quantified using ELISA, the conclusion from which was consistent with the results from Western blot. HMC treatment was adequate to reverse the expressions of IκBα and p-IκBα towards the levels of the healthy controls (Figure 4B).

HMC inhibited the modulators involved in the NF-κB signaling pathway

We then quantified the expression of other modulators in the NF-κB signaling pathway, such as IKKα/β, p-IKKα/β, and p-p65, inside adjuvant vertebral tissues through Western blot and ELISA (Figure 5). From the results of Western blot, the expression of IKKα/β was not significantly altered by different treatments (Figure 5A). The phosphorylation levels of IKKα/β and p65 were elevated in ST group and then reduced to levels similar to sham rabbits in the ST + HMC group. The relative expressions of p-IKKα/β and p-p65 were further verified using ELISA, in which the expressions of both proteins were upregulated by more than two folds in the ST group compared to the sham group (Figure 5B and C). As expected, in ST + HMC group, the expressions of both proteins were markedly suppressed, and no significant difference between the levels of p-IKKα/β in sham group and ST + HMC group was observed (Figure 5C).

Discussion

In the current study, we aimed to investigate the efficacy of HMC in treating tuberculosis *in vivo*. In most cases, rodents are adequate models to study human diseases; however, granulomas with caseous necrosis are not readily formed in tuberculosis rats.[26] Therefore, rodent models are rarely utilized in ST studies. On the other hand, the pathology of tuberculosis in rabbits is similar to that observed in human patients, in which latent tuberculosis can recur when immune balances are disrupted.[26] Thereby, the New Zealand white rabbits were used to evaluate the function of HMC in treating ST in our current study.

Granulomas are described as the aggregation of differentially differentiated macrophages, such as epithelioid cells, foam cells, and Langhans giant cells.[3,13] Multinucleated Langhans giant cells formed via the

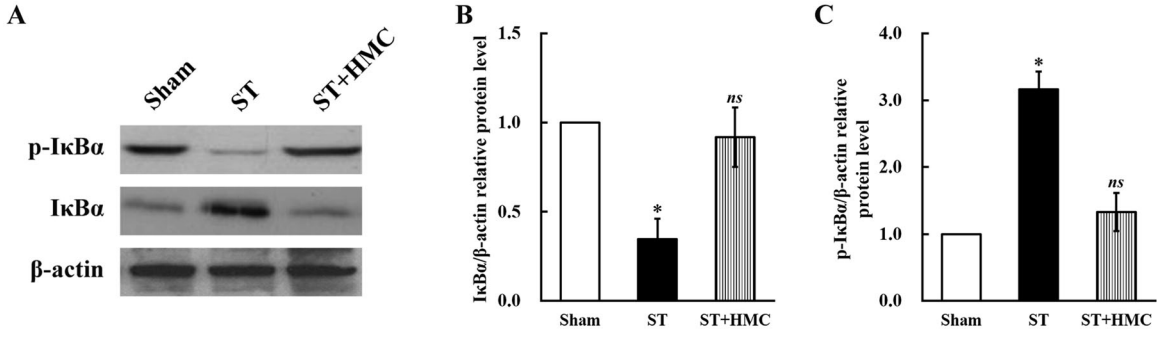

Figure 4 The effects of HMC on degradation and phosphorylation of IκBα. (A) Western blot of protein expressions of IκBα and p-IκBα in lymphocytes. (B) IκBα/β-actin relative expression level. (C) p-IκBα/β-actin relative expression level. *P<0.05 compared to the sham and ST + HMC groups; ns: P>0.05 compared to sham group (n = 10 each group).

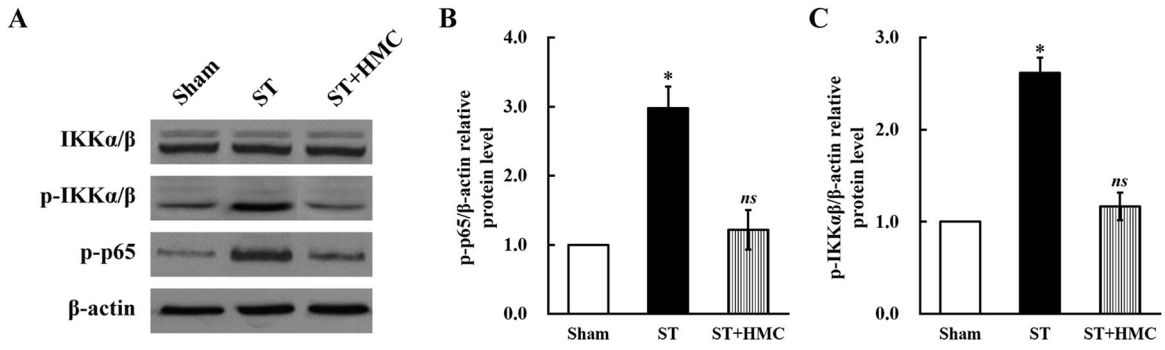

Figure 5 The effects of HMC on phosphorylation of NF-κB subunit p65 and IKKα/β in lymphocytes. (A) Protein expressions of total IKKα/β, p-IKKα/β and p-p65 detected by Western blot. (B) p-p65/β-actin relative expression level. (C) p-IKKα/β/β-actin relative expression level. *P<0.05 compared to the sham and ST + HMC groups; ns: P>0.05 compared to sham group (*n* = 10 each group).

infusion of several macrophages is unique to the granulomas formed in tuberculosis patients.[3] The continuous accumulation of immune cells gives rise to caseous necrosis in the center of granulomas and subsequent liquefaction of the necrosis tissues, which eventually results in irreversible damages.[27] It is therefore essential to inhibit granulomas formation in the treatment of ST. Our findings revealed that the continuous treatments of HMC were adequately effective in inhibiting granulomas formation. Experiments have shown that hesperidin exhibits anti-angiogenesis effects by inhibiting the vascular formation of human umbilical vascular endothelial cells.[28] However, investigations regarding the mechanisms underlying the anti-angiogenesis effects of HMC are still very limited.

The granulation starts with the increased secretion of proinflammatory cytokines and chemokines near the infection site.[10] MCP-1 is one of the most studied chemokines, the lack of which could delay the recruitment of monocytes for 72 hours.[29] Furthermore, the polymorphism of MCP-1 has been shown to alter the susceptibilities of patients to *Mtb* to as high as 6.8 times through regulating the expression of IL-12p40.[15,30] In our previous report, the expression level of MCP-1 was elevated after *Mtb* infection in New Zealand rabbit models.[17] Hence, MCP-1 is a potential target in the treatment of ST. In the present study, we have demonstrated for the first time that HMC treatment suppresses the expression of MCP-1 to a similar level as to normal rabbits.

In previous studies, the expression of NF-κB subtype p65 has been demonstrated to be increased in lymphocytes in New Zealand white rabbits carrying ST.[18] NF-κB is a potent regulator of inflammatory responses in various diseases, and its overexpression could be deleterious. Based on a mathematical model among the cytosolic levels of NF-κB, a certain amount of NF-κB is sufficient to kill bacteria, whereas higher levels of NF-κB might result in aggregation of a greater amount of uninfected macrophages, thereby promoting granulation.[31] It is also reported that the inhibition of NF-κB suppressed the *Mtb* survival in human macrophages by promoting apoptosis and autophagy of infected cells.[32] In our experiments, the *Mtb* induced p65 expressions were markedly prevented by HMC. Thus, HMC is likely to suppress the excessive inflammation during granulation.

We further established that HMC inhibited the activation of an NF-κb signaling pathway in ST rabbits, in which the expression of the inhibitor of κB α (IκBα) was decreased, whereas the phosphorylation of its upstream regulator IκB kinase α/β (IKKα/β) was elevated. The effects of hesperidin and HMC on NF-κB signaling have been reported in mouse models. In a mouse model of diabetes, hesperidin was shown to reduce the NF-κB level,[33] while in a mouse pain model HMC was able to inhibit NF-κB activity.[22] In addition, HMC has also been shown to inhibit proinflammatory cytokines downstream of NF-κB, such as TNF-α, IL-1β, IL-6, and IL-10.[24] In the present study, the serum levels of IL-4 and IL-10 were prominently inhibited by HMC, which had only limited impact on IL-2 and IFN-γ. IL-4 is involved in the activation of type 2 T helper (T_h2) cells. In mice, IL-4 is shown to be highly expressed during the progressive phase of tuberculosis.[34] However, little is known regarding the regulatory effects of HMC on IL-4. IL-10, also known as inhibitors of cytokine synthesis, is able to inhibit the activation of T_h1 cells.[35] In pulmonary tuberculosis, IL-10 is upregulated and the neutralization of which stimulated the proliferation of T-lymphocytes.[36] The inhibition of IL-10 by HMC has been demonstrated in a mouse model of ultraviolet B irradiation-induced skin damage.[24] IFN-γ is a critical proinflammatory cytokine in the activation of macrophages in tuberculosis. In prior reports, higher levels of INF-γ are associated with poor

outcomes in ST patients.[37] IL-2 is another cytokine that promotes the activation, proliferation, and differentiation of most immune cells in response to antigenic activation.[38] All four cytokines are regulated by NF-κB. However, treatments with HMC did not significantly alter the serum levels of IFN-γ and IL-2. Further experiments are necessary to understand the mechanism by which HMC influenced the expression of cytokines in treating ST.

Conclusion

In summary, we examined the potential of HMC as a novel therapeutic option in the treatment of ST. We demonstrated that HMC could effectively prevent granulomas formation, likely by inhibiting the expression of NF-κB and MCP-1, and proinflammatory cytokines such as IL-10 and IL-4.

Disclaimer statements

Contributors None.

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Declaration of interest None.

Conflicts of interest Authors have no conflict of interests to declare.

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